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(71) Applicant *(for all designated States except US):* CRYOLIFE, INC. [US/US]; 2211 New Market Parkway, Suite 142, Marietta, GA 30067 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only) :* CARPENTER, John, F. [US/US]; 4921 Tremont Drive, Marietta, GA 30066 (US).

(74) Agents: HELLER, Paul, H. et al.; Kenyon & Kenyon, One Broadway, New York, NY 10004 (US).

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(54) Title: METHOD FOR STABILIZATION OF BIOMATERIALS

(57) Abstract

A method for stabilizing biomaterials during lyophilization using a two component additive. One component serves as a cryoprotectant such as polyethylene glycol, polyvinyl pyrrolidone, hydroxyethyl starch, dextran or ficoll which protects the protein during freezing. The second component such as sugars, polyhydroxy alcohols, amino acids or methylamine protects the biomaterial during drying.

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METHOD FOR STABILIZATION OF BIOMATERIALS

BACKGROUND

The invention described herein relates to a method of stabilizing biomaterials, especially labile proteins, during drying or freeze-drying using stabilizing components to achieve enhanced protection. Freeze-drying processes have been used with limited degrees of success in protein preservation due to protein denaturation and/or inactivation caused by the freezing or sublimation steps. Even under conditions where the freezing phase of the process is not damaging (i.e., the protein can withstand freezing stress or a cryoprotectant is used), the subsequent removal of water from the sample by sublimation of the frozen sample (drying phase) can lead to irreversible damage.

Carpenter et al, Biochem. Biophys. Acta 923:109-115, 1987 relates to cryoprotectants for proteins which do not stabilize the same proteins during freeze-drying. Additives which have the capacity to protect labile proteins during both freezing and drying can fail to provide freeze-drying stabilization if used under inappropriate conditions (e.g., too high of an initial concentration of additive used; Carpenter and Crowe, Biochemistry, 28:3916-3922, 1989).

Freeze-drying preservation and the uncertainties involved with protein stabilization during freeze-drying make the combined effects of cryopreservation and protein stabilization during freeze-drying largely empirical. Clearly there is a need for a reliable, effective and predictable method of stabilizing

proteins during freeze-drying. This is because any protein product that is insufficiently stable in aqueous solution during distribution, storage, and use will desirably be freeze-dried (lyophilized).

Freeze-dried protein products are seldom composed of pure protein. This is due to the instability of proteins under the stresses encountered during freezing and drying. Stabilizing solutes (excipients) are added to proteins to improve their resistance to damage induced by the acute stresses encountered during freeze-drying and rehydration. In addition, additives are also used to improve the stability of the protein during long-term storage of the dried product. In some cases proteins are inherently stable against acute freeze-drying stress, and only need to be protected during the subsequent storage of the dried product. Human growth hormone and ribonuclease A are examples of this type of protein.

Proteins must withstand the acute effects of freeze-drying and rehydration for storage stability to become significant. It is to this aspect of the art that the current invention is directed. Namely, the invention described herein relates to a method of employing solutes which stabilize labile proteins and other biomaterials during the acute stresses encountered during freeze-drying. Additionally, the compositions of the present invention enhance overall protein recovery after freeze-drying and storage of the dried product. The solute mixture that confers stability against acute stresses during freeze-drying also aids in long-term storage of the protein or other biomaterial.

Damage to freeze-dried proteins is manifested after rehydration, for example, as a loss of protein solubility, aggregation upon rehydration, loss of activity in appropriate bioassays (e.g., stimulated or depressed mitosis or activity of cells induced by growth factors, or antigen binding by an antibody) or in the case of enzymes, a loss of catalytic activity. The latter

parameter is an extremely sensitive measure of protein damage. An enzyme that is fully soluble and has not aggregated may still display a loss of catalytic activity if it has undergone damaging conformational changes. In addition, assays for characterizing catalytic activity are straightforward and are not subjected to the variability and vagaries of bioassays, such as those employed for hormones and growth factors. Hence enzymes are ideal model systems for studies on the stabilization of proteins during freeze drying.

One object of the present invention is to provide a method of stabilizing proteins against both the freezing and drying stresses encountered during lyophilization, preferably by employing a two-component system. Whereas component one or two when used alone may not provide adequate protection of the protein both during freezing and drying, when these components are used in combination, protection during freezing and drying is obtained. Thus with embodiments of this method, synergistic protection of the protein can be realized during freeze-drying, with component one providing primary stabilization against freezing and component two primarily protecting during the drying phase. One advantage of the present invention is to obtain freeze-drying stabilization by employing solutes that would not normally be expected to protect labile proteins during freeze-drying.

An object of the present invention is to speed and simplify development of freeze-drying protocols for the stabilization of labile proteins. These and other objectives will be apparent to those of ordinary skill in the art from the teachings herein.

SUMMARY OF THE INVENTION

An improved method for stabilizing biomaterials, especially labile proteins, during freeze-drying is disclosed, wherein stabilization is realized employing an additive system, preferably a two-component

system. In embodiments of the invention one component may serve as a cryoprotectant to protect the protein against damage due to freezing, while conferring minimal protection during the subsequent drying phase. Component two may be selected and employed to confer minimal protection during freezing, and when used alone affords no protection or is damaging to the protein during drying. Thus, with this method, protection is realized during freeze-drying, with component one providing stabilization against damage due to freezing and component two protecting during the drying phase when it is employed in the solute mixture of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

The invention is described in detail in conjunction with the accompanying drawings.

Figure 1.

A. Comparison of the influence of polyethylene glycol on the stability of lactate dehydrogenase ("LDH") during freeze-thawing versus during freeze-drying and rehydration.

B. Comparison of the influence of trehalose alone versus trehalose in combination with 1% (wt/vol.) polyethylene glycol on the stability of lactate dehydrogenase during freeze-thawing.

C. Comparison of the influence of trehalose alone versus trehalose in combination with 1% (wt/vol.) polyethylene glycol on the stability of lactate dehydrogenase during freeze-drying and rehydration.

Figure 2.

A. Comparison of the influence of lactose alone versus lactose in combination with 1% (wt/vol) polyethylene glycol on the stability of lactate dehydrogenase during freeze-thawing.

B. Comparison of the influence of lactose alone versus lactose in combination with 1% (wt/vol)

polyethylene glycol on the stability of lactate dehydrogenase during freeze-drying and rehydration.

Figure 3.

A. Comparison of the influence of glucose alone versus glucose in combination with 1% (wt/vol) polyethylene glycol on the stability of lactate dehydrogenase during freeze-thawing.

B. Comparison of the influence of glucose alone versus glucose in combination with 1% (wt/vol) polyethylene glycol on the stability of lactate dehydrogenase during freeze-drying and rehydration.

Figure 4.

A. Comparison of the influence of glycine alone versus glycine in combination with 1% (wt/vol) polyethylene glycol on the stability of lactate dehydrogenase during freeze-thawing.

B. Comparison of the influence of glycine alone versus glycine in combination with 1% (wt/vol) polyethylene glycol on the stability of lactate dehydrogenase during freeze-drying and rehydration.

Figure 5.

A. Comparison of the influence of mannitol alone versus mannitol in combination with 1% (wt/vol) polyethylene glycol on the stability of lactate dehydrogenase during freeze-thawing.

B. Comparison of the influence of mannitol alone versus mannitol in combination with 1% (wt/vol) polyethylene glycol on the stability of lactate dehydrogenase during freeze-drying and rehydration.

DETAILED DESCRIPTION

Freeze-drying involves lyophilization and refers to the process by which an aqueous solution of a protein and additives is frozen, after which the water is removed by sublimation at low pressure. Subsequent rehydration of the dried protein preparation is typically necessary to use the protein and to assay the effect of

the processing steps on protein activity. The drying phase typically involves all stages of the sublimation process following freezing.

Excipients and stabilizing solutes or additives are compounds that are added to the protein solution or suspension (broadly referred to herein as a "protein solution") prior to freeze-drying to improve the stability of the protein to the freeze-drying cycle, and to improve the storage stability of the dried product.

The invention described herein relates to a method of employing preferably a two-component mixture of solutes to stabilize labile proteins against the stresses encountered by freeze-drying. However, the method also aids in overall protein recovery after freeze-drying and storage of the dried product. That is, the solutes that confer stability against acute stresses also aid in the long-term storage of the protein.

Component one, used to stabilize the protein, comprises a polymer which provides at least some cryoprotection for the protein. The cryoprotectant is preferably a compound that has the capacity to protect the protein during freezing and subsequent thawing or during the freezing phase of a freeze-drying process. Some cryoprotectants also protect during the subsequent drying step of freeze-drying. Preferred examples of polymers that can serve as cryoprotectants for proteins include: polyethylene glycol, polyvinyl pyrrolidone, hydroxyethyl starch, dextran and ficoll. The polymers used herein can include compounds having a molecular weight of greater than about 500 daltons.

Polyethylene glycol is a preferred polymer useful as component one because it is commonly used during protein purification and thus it often present in solution with a protein to be freeze-dried. Polyethylene glycol is useful in that t prevents the protein from sticking to the surface of the container in which it is present. A preferred level of polyethylene glycol is from

about 0.5 to about 20%(w/v) of the suspension.

Although thawing is not typically a step in the freeze-drying process, by thawing a frozen sample one can assess the effects of the freezing step, independent of a subsequent drying step, on the stability of the protein.

Component two is a compound that when used alone may provide minimal if any protection for the protein during either freezing or drying, and may actually potentiate the protein damage when used alone. Examples of compounds that can be used as component two include:

(1) sugars, (e.g., trehalose, lactose, sucrose, glucose, galactose, maltose, mannose and fructose);

(2) polyhydroxy alcohols (e.g., mannitol, sorbitol and inositol);

(3) amino acids (e.g., glycine, alanine, proline and lysine); and

(4) methylamines (e.g., trimethylamine-N-oxide, betaine and sarcosine).

Some compounds suitable for use as the second component protect freeze-dried proteins under certain conditions, such as at specific initial concentrations (e.g., the influence of trehalose on freeze-dried phosphofructokinase; Carpenter and Crowe, Biochemistry 28:3916-3922, 1989), whereas others appear to be virtually ineffective at protecting labile proteins during freeze-drying (e.g., the influence of glycine on freeze-dried phosphofructokinase; Carpenter et al., Biochem, Biophys. Acta, 923: 109-115, 1987). However, when used in combination with component one, component two protects the protein during the drying phase of the freeze-drying process.

It has been found that compounds having substituents, e.g. hydroxyl, amino or substituted amines, capable of hydrogen bonding to the protein are useful as component two in the present invention. While not wishing

to be bound by this theory, it may be that such compounds may serve to replace water which is removed from the protein during drying, and thereby prevent the protein from aggregating, refolding, or undergoing some other transformation that reduces its activity.

When a sugar is used as component two, a preferred level is from about 1 to about 300 mM. One advantage of the present invention, is that when a polymeric cryoprotectant is used as component one, however, freeze-drying stabilization is not limited to sugars. Compounds such as polyhydroxy alcohols, amino acids and methylamines are surprisingly effective at stabiLizing proteins during the drying phase.

It should be apparent to one skilled in the art that during the practice of the present invention, component one can be comprised of one or more of the cryoprotectant solutes defined above. Similarly, component two can be comprised of one or mo:-e of the above defined solutes.

In addition, one skilled in the art will recognize that the invention described herein can also be used to stabilize other biological materials such as viruses or human, animal, or bacterial cells during freeze drying.

In embodiments of the present invention, proteins, such as a growth factors, hormones, antibodies, antigens, enzymes, clotting factors, structural proteins and complement factors can be combined with components one and two as described herein and subjected to freeze-drying. Specifically, such proteins would include: Erythropoietin; Factor VIII; G-Colony Stimulating Factor; GM-Colony Stimulating Factor, Antithrombin III; insulin; Epidermal Growth Factor; Acidic Fibroblast Growth Factor; Basic Fibroblast Growth Factor; Interleukins (IL 1 alpha, IL 1 beta, IL 2, IL 3, IL 4, IL 6, IL 7, IL 8, IL 9, IL 10); Tumor Necrosis Factor-alpha; Tumor Necrosis Factor-beta; Interferon-alpha; Interferon-gamma; Transforming

Growth Factor-beta; Tissue Plasminogen Activator; Platelet-derived Growth Factor; Urokinase; Streptokinase; Peroxidase; RNA polymerase; T7 DNA polymerase; Taq DNA polymerase; Fibrinogen; Thrombin; Alcohol dehydrogenase; Alkaline phosphatase; Arginase; Ascorbate oxidase; Cholesterol esterase; Cholinesterase; Collagenase; DNase I; DNase II; Enterokinase; Glucose-6-phosphate dehydrogenase; Glucose oxidase; Glucose Isomerase; Glutamate dehydrogenase; Glyceraldehyde-3-phosphate dehydrogenase; Hexokinase; Lactate Dehydrogenase; Malate dehydrogenase; PEP carboxylase; RNase A; Soybean trypsin inhibitor; Urease; Xanthine oxidase; Superoxide dismutase; Fibronectin; Restriction endonucleases; Reverse transcriptase, AMV; Reverse transcriptase, M-MuLV; Monoclonal antibodies: OKT3, HA-1A, BMA 031, CAMPATH-1, anti-TAC, etc.; Polyclonal antibodies; Human IgM; and Human IgG.

Similarly, a labile biomaterial such as a liposome, cell organelle, vaccine, virus, bacteria, eukaryotic cell, platelet, living-cell viral vaccine, other subcellular element, genetic material or other biomaterial can be freeze-dried according to the embodiments described herein.

An assay of structural or functional integrity after rehydration would indicate stabilization of the protein or other biomaterial over that which would occur had the two component system not been used. Furthermore, the presence of the additives would increase the stability of a protein or other biomaterial once it has been rehydrated.

A further embodiment of the present invention involves the use of the two component system described herein to stabilize proteins and other biomaterials against the detrimental effects of alternative means of drying, including spray drying or vacuum centrifugation.

EXAMPLE 1

Rabbit muscle lactate dehydrogenase (M isozyme,

99+% homogeneous) was purchased as a crystalline suspension in ammonium sulfate (Sigma; St. Louis, Mo). Prior to each experiment the lactate dehydrogenase ("LDH") was dialyzed (4°C) for several hours against 10 mM potassium phosphate buffer (pH 7.5 at 23°C). An aliquot of the stock enzyme was added to the appropriate preparation of polyethylene glycol (MW 8000) (prepared in the 10 mM potassium phosphate buffer) to a final LDH concentration of 25 μg/ml and 10% (wt/vol) of polyethylene glycol (PEG). Samples of various concentrations of PEG were obtained by diluting the above mixture with a solution containing 25 μg/ml of LDH in the buffer alone. In order to test a two-component system in which PEG served as component one and trehalose served as component two, 25 μg/ml of LDH was prepared in the buffer containing 1% (wt/vol) PEG and 100 mM trehalose. Various concentrations of trehalose were obtained by diluting this mixture with a buffer solution containing 25 μg/ml LDH and 1% PEG without trehalose.

FREEZE-THAW EXPERIMENTS

A 75 μl aliquot of a given mixture was transferred to a 1.5 ml polypropylene Eppendorf test tube. A 10 μl sample was removed and assayed for LDH catalytic activity. This served as the pre-treatment control value. LDH catalytic activity was measured at 25°C. The 2.0 ml reaction mixture contained 80 mM Tris/Hcl buffer (pH 7.5), 100 mM KCl, 2 mM pyruvate, and 0.15 mM NADH. The remaining 65 μl aliquot in the test tube was frozen by immersion into liquid nitrogen for 30 seconds. Samples were then thawed at room temperature, immediately mixed and assayed for residual activity. The results are expressed as the percentage of the pre-treatment activity recovered after thawing.

FREEZE-DRYING EXPERIMENTS

Triplicate 75 μl aliquots (which were identical

to the sample used for freeze-thawing) were placed into Eppendorf test tubes. The samples were frozen in liquid nitrogen and then placed on a VirTis lyophilizer for a minimum of 16 hours at 15-40 mTorr. The freeze-dried samples were rehydrated in distilled water, and residual LDH activity was assayed. The results are expressed as the percentage of the pre-treatment activity recovered after rehydration.

The results in Fig. 1A demonstrate that PEG completely protects LDH from inactivation during freeze-thawing. Essentially full activity is recovered at all PEG concentrations tested. By contrast, PEG provides essentially no stabilization to the enzyme during freeze-drying. These results indicate that PEG is an ideal cryoprotectant for proteins, but that when used alone it does not have the capacity to stabilize freeze-dried proteins.

The influence of trehalose (either alone or in combination with 1% PEG) on LDH stability during freeze-thawing is shown in Fig. 1B. The combination of trehalose and 1% PEG provides almost full protection for the enzyme during freeze-thawing. By contrast, trehalose alone provides almost no protection during freeze-thawing.

Fig. 1C shows the effect of trehalose alone and trehalose in combination with 1% PEG on the stability of LDH during freeze-drying. Trehalose alone provides no stabilization and actually appears to cause additional damage to the enzyme during freeze-drying. In contrast, when trehalose is used in combination with 1% PEG (which alone provides only minimal protection) almost 100% of the LDH activity is retained after freeze-drying.

These results support synergistic stabilization of proteins during freeze-drying. In this example, PEG is the solute comprising component one, which fully protects against damage induced by freezing, while conferring minimal if any protection during the subsequent drying phase. Trehalose is the solute comprising component two,

which confers minimal protection during freezing, and when used alone affords no protection and/or is damaging during drying. Thus, when either component is used alone there is at best minimal stabilization of labile proteins during freeze-drying. Surprisingly, however, when used in combination with PEG, trehalose provides almost complete protection during the drying phase. Thus, with this method synergistic protection is realized during freeze-drying.

EXAMPLE 2

Samples were prepared and treated as described in ExampLe 1, except that lactose was substituted for trehalose and used as component two. The influence of lactose (either alone or in combination with 1% PEG) on LDH stability during freeze-thawing is shown in Fig. 2A. The combination of lactose and 1% PEG provides protection for the enzyme during freeze-thawing. By contrast, lactose alone provides almost no protection during freeze-thawing.

Fig. 2B shows the effect of lactose alone and lactose in combination with 1% PEG on the stability of LDH during freeze-drying. Lactose alone provides no stabilization and appears to cause additional damage to the enzyme during freeze-drying. By contrast, when lactose is used in combination with 1% PEG (which alone provides only minimal protection) the LDH is stabilized during freeze-drying.

Synergistic protection by the two components is realized during freeze-drying, with PEG providing stabilization against damage during freezing and lactose protecting during the drying phase.

EXAMPLE 3

Samples were prepared and treated as described in Example 1, except that glucose was substituted for trehalose and served as component two. The influence of glucose (either alone or in combination with 1% PEG) on LDH stability during freeze-thawing is documented in Fig.

3A. The combination of glucose and 1% PEG provides protection for the enzyme during freeze-thawing.

Fig. 3B shows the effects of glucose alone and glucose in combination with 1% PEG on the stability of LDH during freeze-drying. Glucose alone provides no stabilization and actually appears to cause additional damage to the enzyme during freeze-drying. By contrast, when glucose is used in combination with 1% PEG (which alone provide only minimal protection) the LDH is stabilized during freeze-drying.

Synergistic protection by the two components is therefore realized during freeze-drying, with PEG providing stabilization against damage during freezing and glucose protecting during the drying phase.

EXAMPLE 4

Samples were prepared and treated as described in Example 1, except that glycine was substituted for trehalose as component 2. The influence of glycine (either alone or in combination with 1% PEG) on LDH stability during freeze-thawing is shown in Fig. 4A. The combination of glycine and 1% PEG provides protection for the enzyme during freeze-thawing. By contrast, glycine alone provides almost no protection during freeze-thawing.

Fig. 4B shows the effects of glycine alone and in combination with 1% PEG on the stability of LDH during freeze-drying. Glycine alone provides no stabilization and actually fosters additional damage to the enzyme during freeze-drying. By contrast, when glycine is used in combination with 1% PEG (which alone provide only minimal protection) the LDH is greatly stabilized during freeze-drying.

Synergistic protection is realized during freeze-drying, with PEG providing stabilization against damage during freezing and glycine protection during the drying phase.

EXAMPLE 5

Samples were prepared and treated as described in Example 1. Mannitol was substituted for trehalose and served as component two. The influence of mannitol (either alone or in combination with 1% PEG) on LDH stability during freeze-thawing is shown in Fig. 5A. The combination of mannitol and 1% PEG provides protection for the enzyme during freeze-thawing. By contrast, mannitol alone provides almost no protection during freeze-thawing.

Fig. 5B shows the effects of mannitol alone and in combination with 1% PEG on stability of LDH during freeze-drying. Mannitol alone provides no stabilization and actually fosters additional damage to the enzyme during freeze-drying. By contrast, when mannitol is used in combination with 1% PEG (which alone provide only minimal protection) the LDH is greatly stabilized during freeze-drying.

Synergistic protection by the two components is realized during freeze-drying, with PEG (component 1) providing stabilization against damage during freezing and mannitol (component 2) protecting during the drying phase.

CLAIMS

1. A method for stabilizing a protein during freezing and drying of a solution of the protein comprising freezing and drying the protein solution in the presence of polyethylene glycol and a sugar.

2. A method according to claim 1 wherein the sugar is selected from the group consisting of trehalose, lactose, sucrose, glucose, galactose, maltose, mannose and fructose.

3. A method according to claim 2 wherein said polyethylene glycol is present in the protein solution at a level of about 0.5 to about 20% weight/volume of the solution and said sugar is present in the protein solution at a level of from about 1 to about 300 mM.

4. A method for stabilizing a protein during freezing and drying of a solution of the protein comprising freezing and drying the protein solution in the presence of polyethylene glycol and a polyhydroxy alcohol.

5. A method according to claim 4 wherein the polyhydroxy alcohol is selected from the group consisting of mannitol, sorbitol and inositol.

6. A method for stabilizing a protein during freezing and drying of a solution of the protein comprising freezing and drying the protein solution in the presence of polyethylene glycol and at least one amino acid.

7. A method according to claim 6 wherein the amino acid is selected from the group consisting of glycine, alanine, proline and lysine.

8. A method for stabilizing a protein during freezing and drying of a solution of the protein comprising freezing and drying the protein solution in the presence of polyethylene glycol and a methylamine.

9. A method according to claim 8 wherein the methylamine is selected from the group consisting of trimethylamine-N-oxide, betaine and sarcosine.

10. A method for stabilizing a protein during freezing and drying of a suspension of the protein comprising freezing and drying the protein solution in the presence of a cryoprotectant and at least one further compound selected from the group consisting of sugars, polyhydroxy alcohols, amino acids and methylamines, wherein neither said cryoprotectant nor said further compound when used alone is capable of stabilizing said protein during freeze drying.

11. A method according to claim 10 wherein said cryoprotectant is selected from the group consisting of polyvinyl pyrrolidone, hydroxyethyl starch, dextran and ficoll.

12. A method for stabilizing a protein during freezing and drying of a solution of the protein comprising freezing and drying the protein solution in the presence of a cryoprotectant and at least one further compound capable of hydrogen bonding to the protein.

13. A method according to claim 12 wherein said cryoprotectant is selected from the group consisting of polyethylene glycol, polyvinyl pyrrolidone, hydroxyethyl starch, dextran and ficoll.

14. A method according to claim 1 wherein said freezing and drying comprises freezing the protein

solution and thereafter drying the solution by sublimation.

15. A method for stabilizing a biomaterial during drying of a solution of the biomaterial comprising drying the biomaterial solution in the presence of a cryoprotectant selected from the group consisting of polyethylene glycol, polyvinyl pyrrolidone, hydroxyethyl starch, dextran and ficoll and at least one second component selected from the group consisting of sugars, amino acids, polyhydroxy alcohols and methylamines.

16. A method according to claim 15 wherein said biomaterial is selected from the group consisting of liposomes, cell organelles, vaccines, viruses, bacteria, eukaryotic cells, platelets, living-cell viral vaccines, other subcellular elements, and genetic materials.

17. A composition suitable for freezing and thereafter drying a biomaterial comprising the biomaterial, polyethylene glycol and a sugar.

18. A composition according to claim 17 wherein said biomaterial is selected from the group consisting of liposomes, cell organelles, vaccines, viruses, bacteria, eukaryotic cells, platelets, living-cell viral vaccines, other subcellular elements, and genetic materials.

19. A composition according to claim 17 wherein said biomaterial is a protein.

20. A composition according to claim 19 wherein said protein is a growth factor, hormone, antibody, antigen, enzyme, clotting factor, structural protein or complement factor.

21. A composition according to claim 19 wherein

said protein is selected from the group consisting of Erythropoietin; Factor VIII; G-Colony Stimulating Factor; GM-Colony Stimulating Factor, Antithrombin III; Insulin; Epidermal Growth Factor; Acidic Fibroblast Growth Factor; Basic Fibroblast Growth Factor; Interleukin (IL 1 alpha, IL 1 beta, IL 2, IL 3, IL 4, IL 6, IL 7, IL 8, IL 9, IL 10); Tumor Necrosis Factor-alpha; Tumor Necrosis Factor-beta; Interferon-alpha; Interferon-gamma; Transforming Growth Factor-beta; Tissue Plasminogen Activator; Platelet-derived Growth Factor; Urokinase; Streptokinase; Peroxidase; RNA polymerase; T7 DNA polymerase; Taq DNA polymerase; Fibrinogen; Thrombin; Alcohol dehydrogenase; Alkaline phosphatase; Arginase; Ascorbate oxidase; Cholesterol esterase; Cholinesterase; Collagenase; DNase I; DNase II; Enterokinase; Glucose-6-phosphate dehydrogenase; Glucose oxidase; Glucose Isomerase; Glutamate dehydrogenase; Glyceraldehyde-3-phosphate dehydrogenase; Hexokinase; Lactate dehydrogenase; Malate dehydrogenase; PEP carboxylase; RNase A; Soybean trypsin inhibitor; Urease; Xanthine oxidase; Superoxide dismutase; Fibronectin; Restriction endonucleases; Reverse transcriptase, AMV; Reverse transcriptase, M-MuLV; Monoclonal antibodies: OKT3, HA-1A, BMA 031, CAMPATH-1, anti-TAC, etc.; Polyclonal antibodies; Human IgM; and Human IgG.

22. A composition according to claim 17 wherein the sugar is selected from the group consisting of trehalose, lactose, sucrose, glucose, galactose, maltose, mannose and fructose.

23. A composition according to claim 22 comprising about 0.5 to about 20% (w/v) polyethylene glycol and about 1 to about 300 mM sugar.

24. A composition suitable for freezing and thereafter drying a biomaterial comprising the

biomaterial, polyethylene glycol and a polyhydroxy alcohol.

25. A composition according to claim 24 wherein said polyhydroxy alcohol is selected from the group consisting of mannitol, sorbitol and inositol.

26. A composition suitable for freezing and thereafter drying a biomaterial comprising the biomaterial, polyethylene glycol and at least one amino acid.

27. A composition according to claim 26 wherein said amino acid is selected from the group consisting of glycine, alanine, proline and lysine.

28. A composition suitable for freezing and thereafter drying a biomaterial comprising the biomaterial, polyethylene glycol and a methylamine.

29. A composition according to claim 28 wherein said methylamine is selected from the group consisting of trimethylamine-N-oxide, betaine and sarcosine.

30. A composition suitable for freezing and thereafter drying a biomaterial comprising the biomaterial, a cryoprotectant and a further compound selected from the group consisting of sugars, polyhdroxy alcohols, amino acids and methylamines.

31. A composition according to claim 30 wherein said cryoprotectant is selected from the group consisting of polyvinyl pyrrolidone, hydroxyethyl starch, dextran and ficoll.

32. A composition suitable for freezing and thereafter drying a protein comprising the protein, a

cryoprotectant and at least one further compound capable of hydrogen bonding to the protein.

33. A composition according to claim 32 wherein said cryoprotectant is selected from the group consisting of polyethylene glycol, polyvinyl pyrrolidone, hydroxyethyl starch, dextran and ficoll.

FIG. 1A

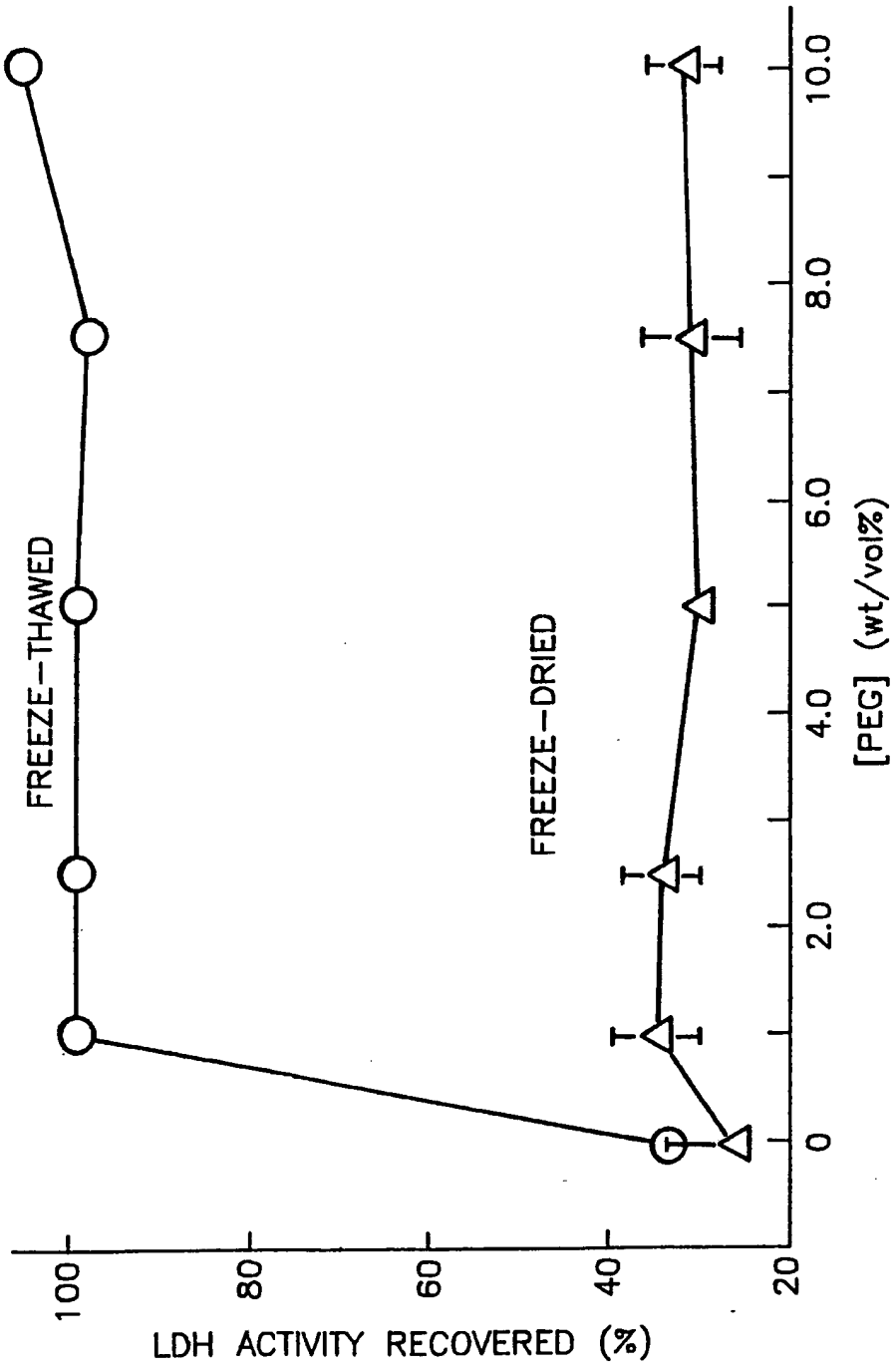

FREEZE-THAWED
FREEZE-DRIED
LDH ACTIVITY RECOVERED (%)
100
80
60
40
20
0
2.0
4.0
6.0
8.0
10.0
[PEG] (wt/vol%)

FIG. 1B

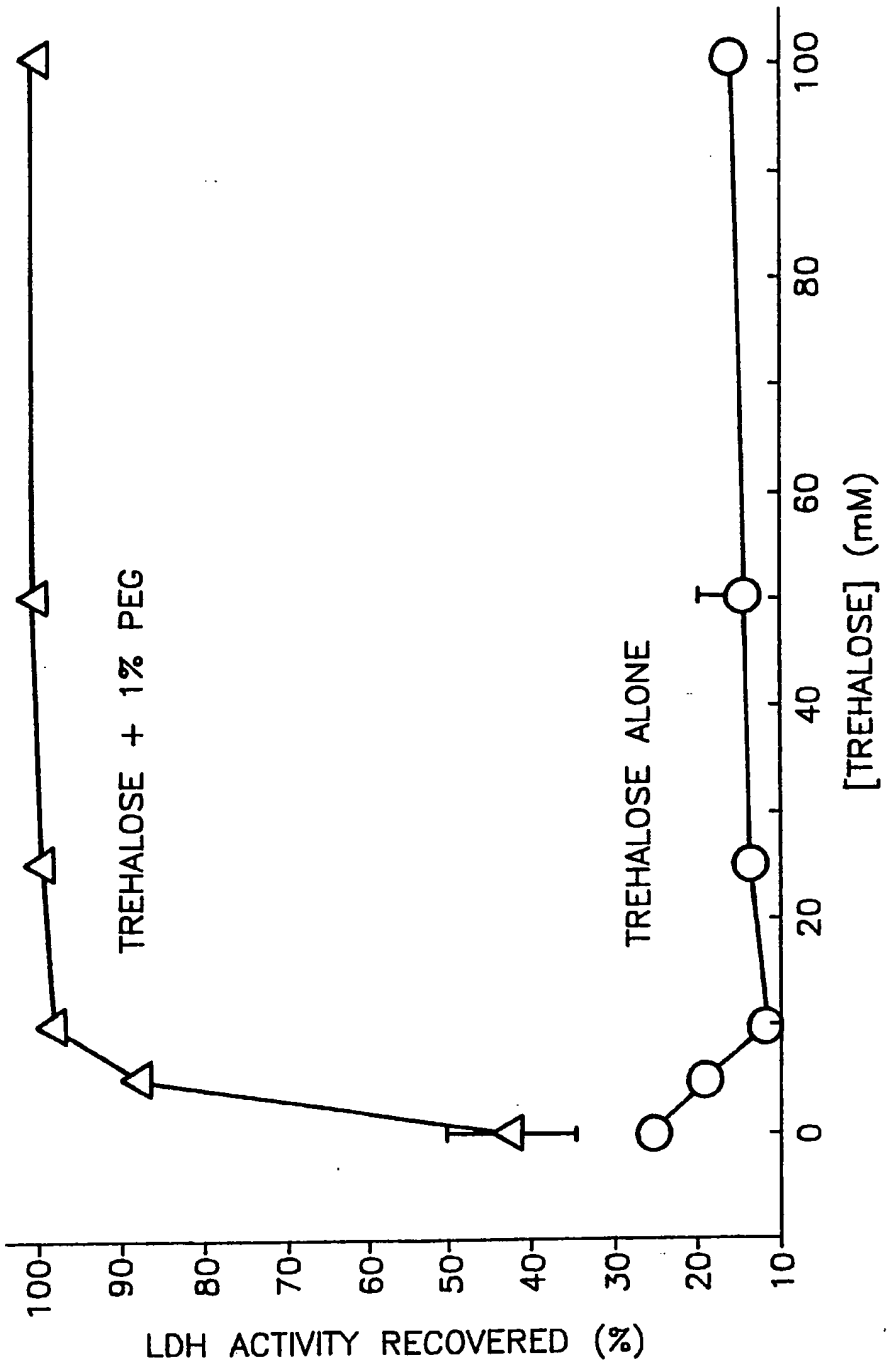

TREHALOSE + 1% PEG
TREHALOSE ALONE
LDH ACTIVITY RECOVERED (%)
[TREHALOSE] (mM)
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
100

FIG. 1C

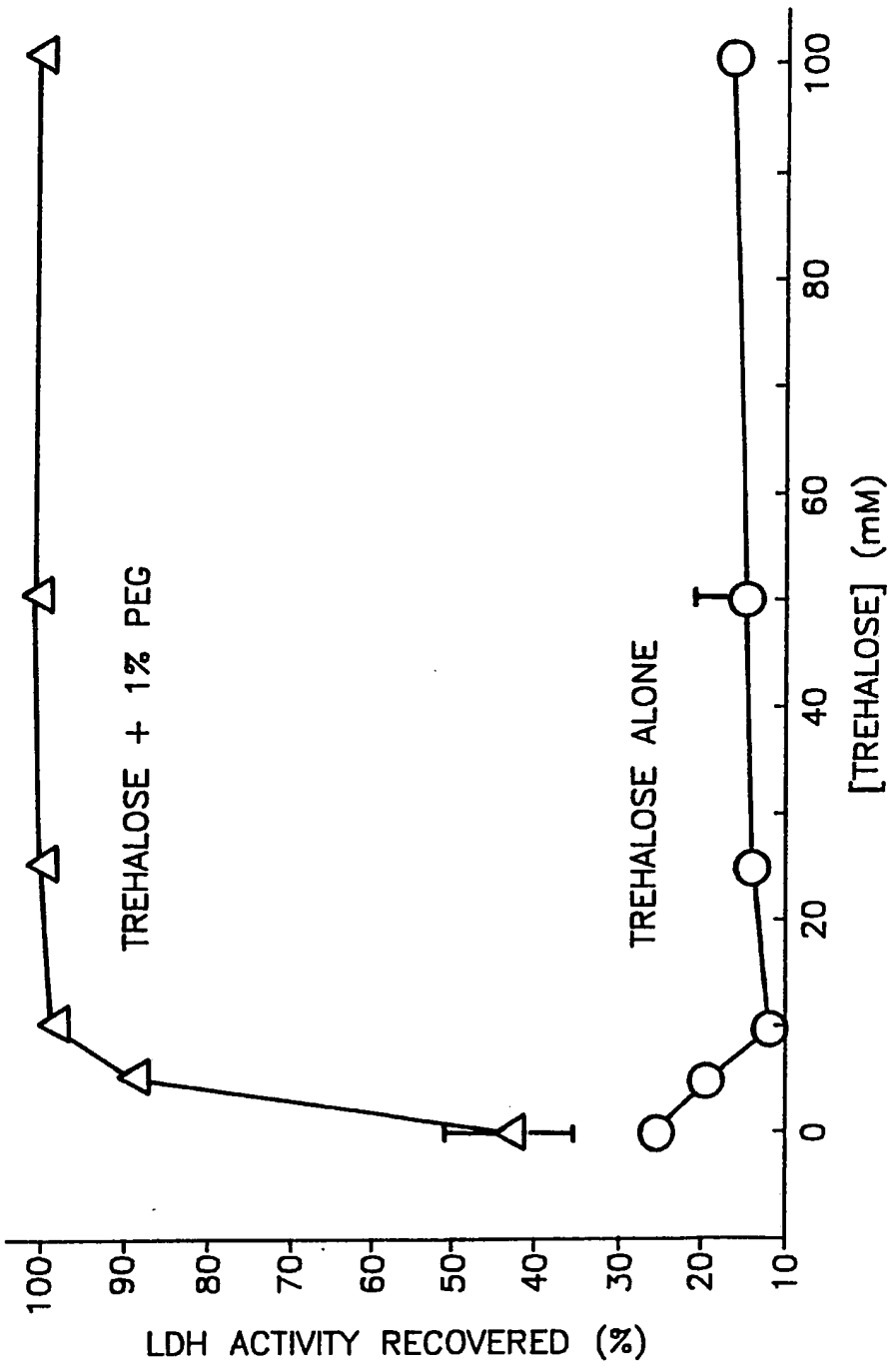

TREHALOSE + 1% PEG
TREHALOSE ALONE
LDH ACTIVITY RECOVERED (%)
[TREHALOSE] (mM)
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
100

FIG. 2A

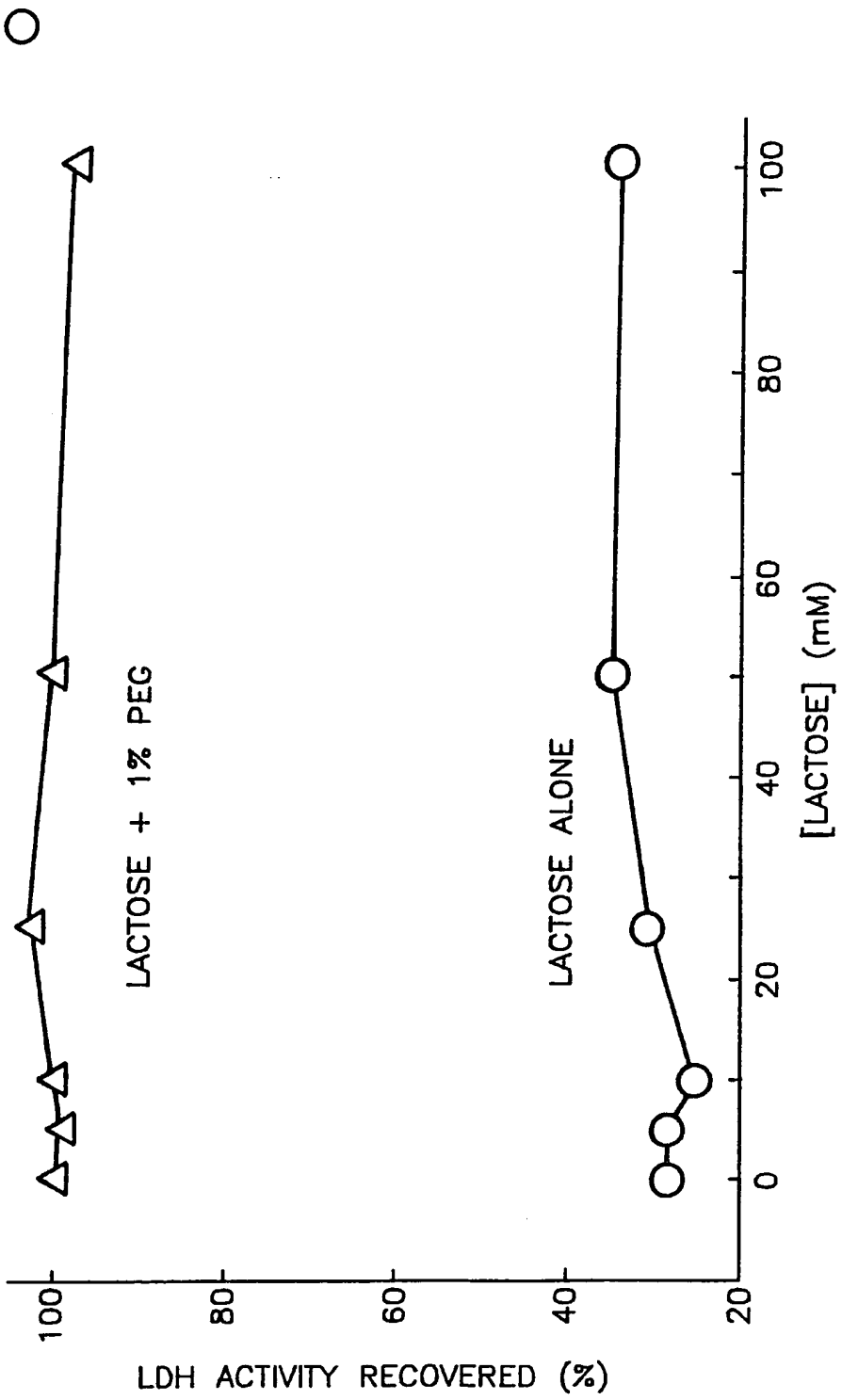

LACTOSE + 1% PEG
LACTOSE ALONE
LDH ACTIVITY RECOVERED (%)
100
80
60
40
20
0
20
40
60
80
100
[LACTOSE] (mM)

FIG. 2B

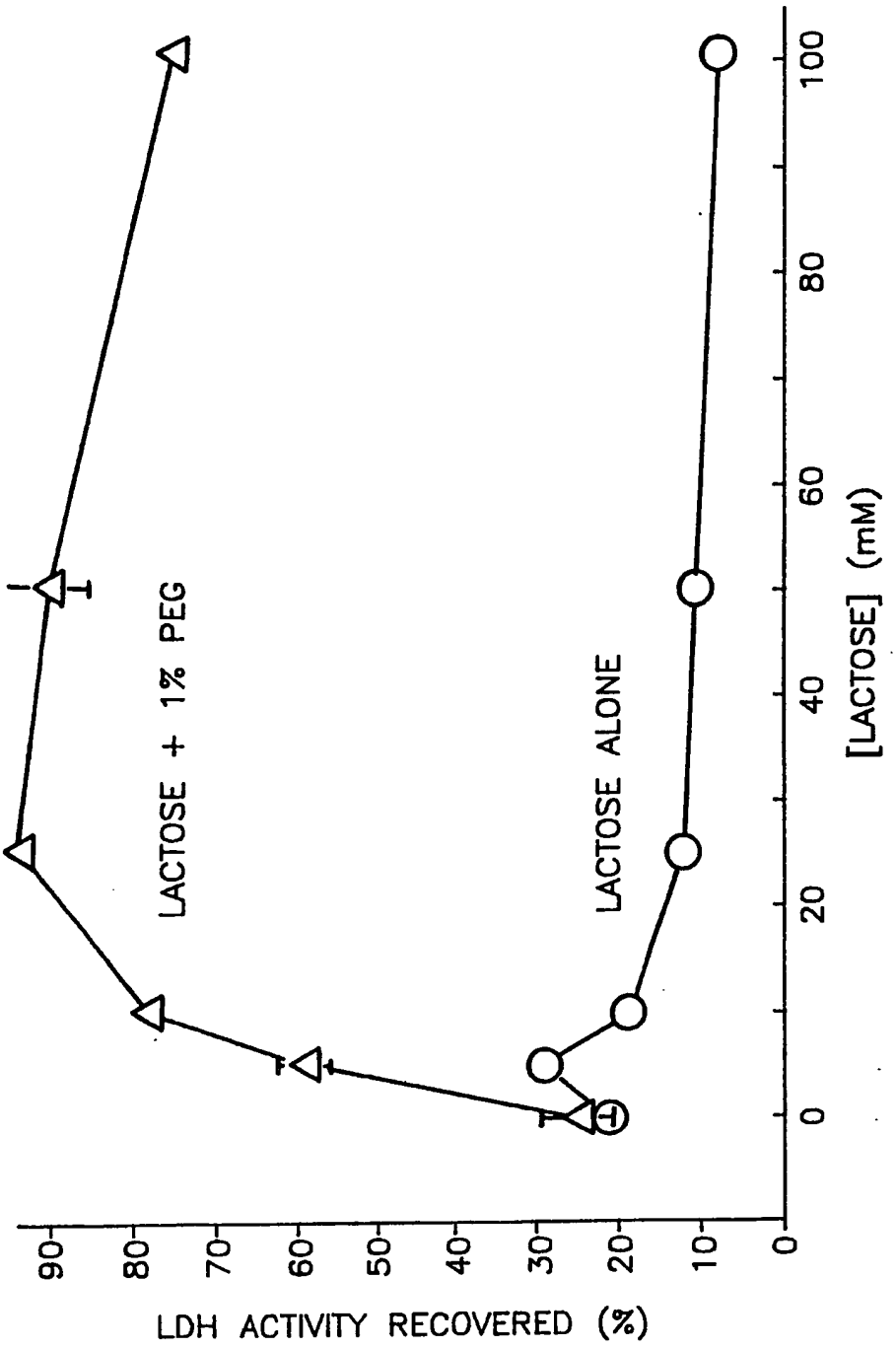

LACTOSE + 1% PEG
LACTOSE ALONE
LDH ACTIVITY RECOVERED (%)
[LACTOSE] (mM)
0
10
20
30
40
50
60
70
80
90
0
20
40
60
80
100

FIG. 3A

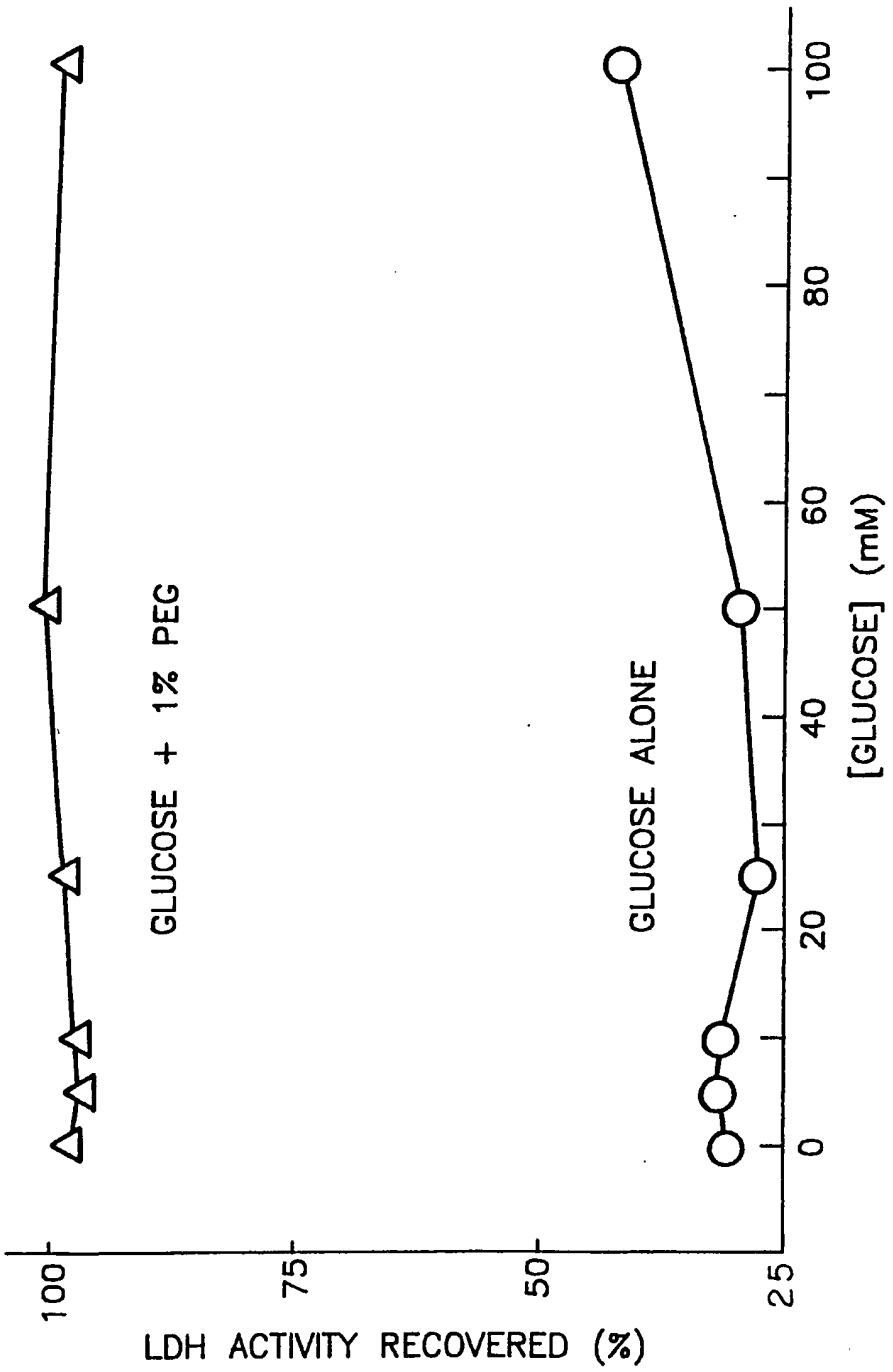

GLUCOSE + 1% PEG
GLUCOSE ALONE
LDH ACTIVITY RECOVERED (%)
100
75
50
25
0
20
40
60
80
100
[GLUCOSE] (mM)

FIG. 3B

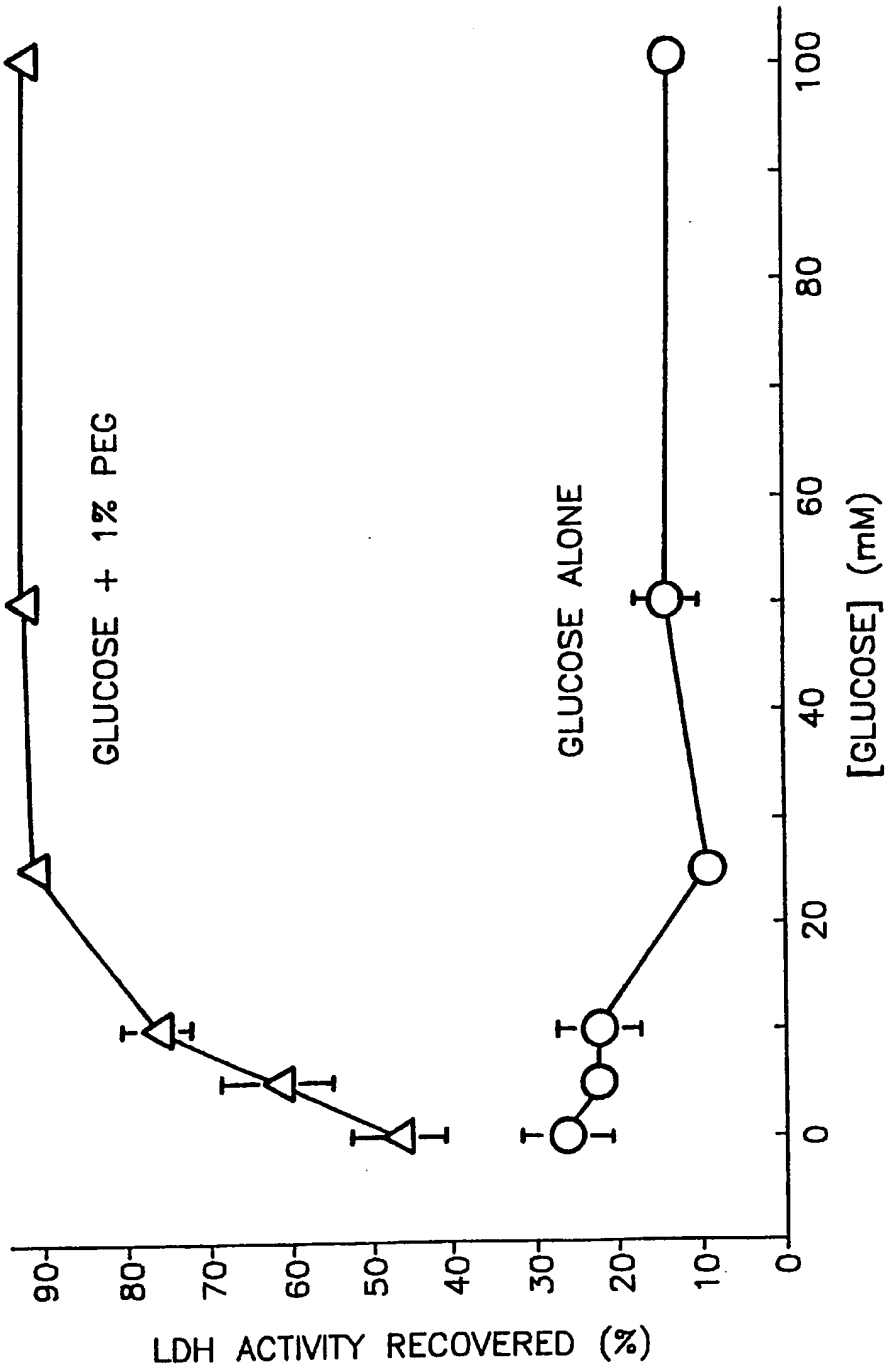

GLUCOSE + 1% PEG
GLUCOSE ALONE
LDH ACTIVITY RECOVERED (%)
[GLUCOSE] (mM)
0
10
20
30
40
50
60
70
80
90
0
20
40
60
80
100

FIG. 4A

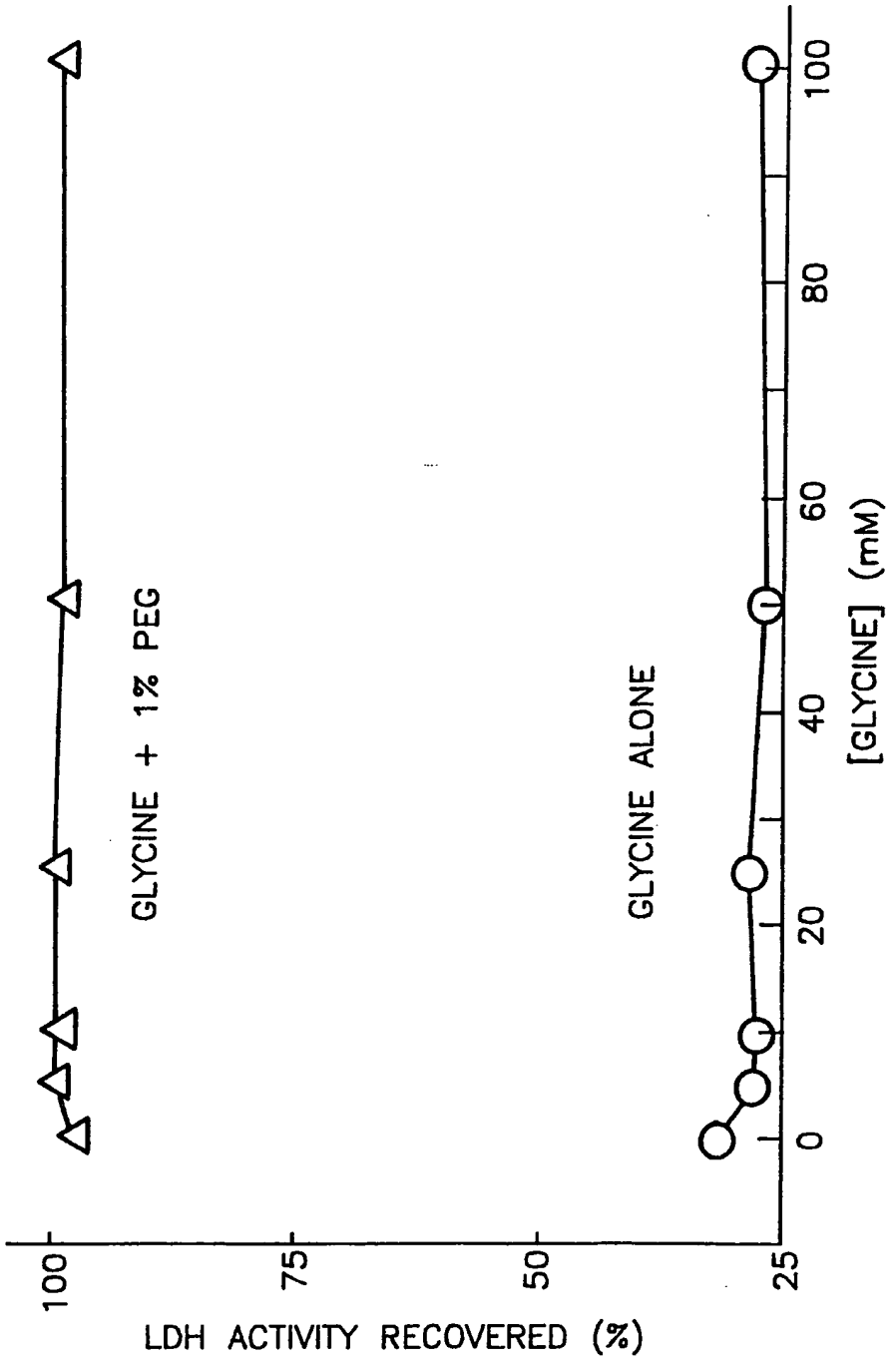

GLYCINE + 1% PEG
GLYCINE ALONE
100
75
50
25
LDH ACTIVITY RECOVERED (%)
0
20
40
60
80
100
[GLYCINE] (mM)

FIG. 4B

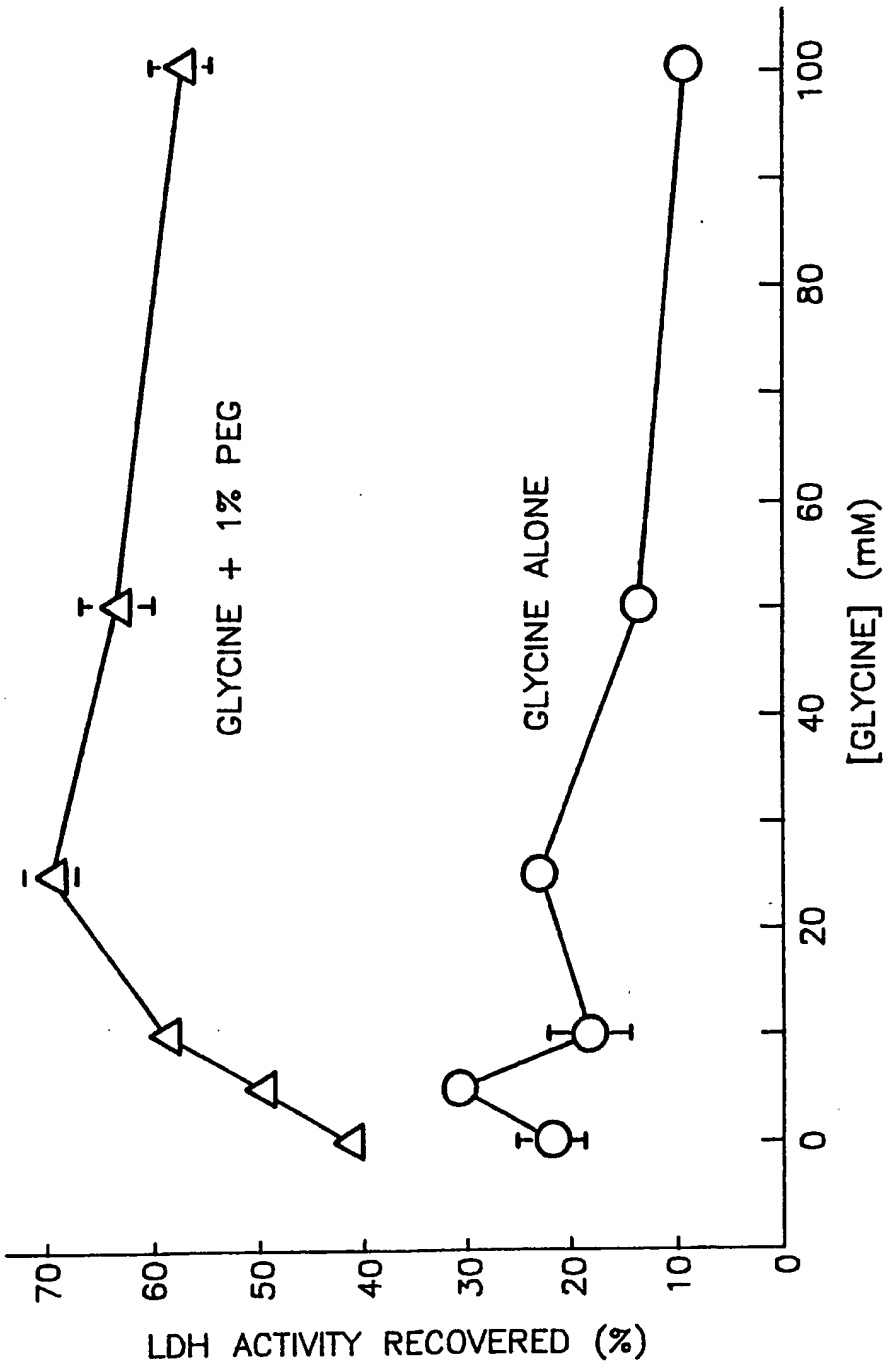

GLYCINE + 1% PEG
GLYCINE ALONE
LDH ACTIVITY RECOVERED (%)
[GLYCINE] (mM)
0
10
20
30
40
50
60
70
0
20
40
60
80
100

FIG. 5A

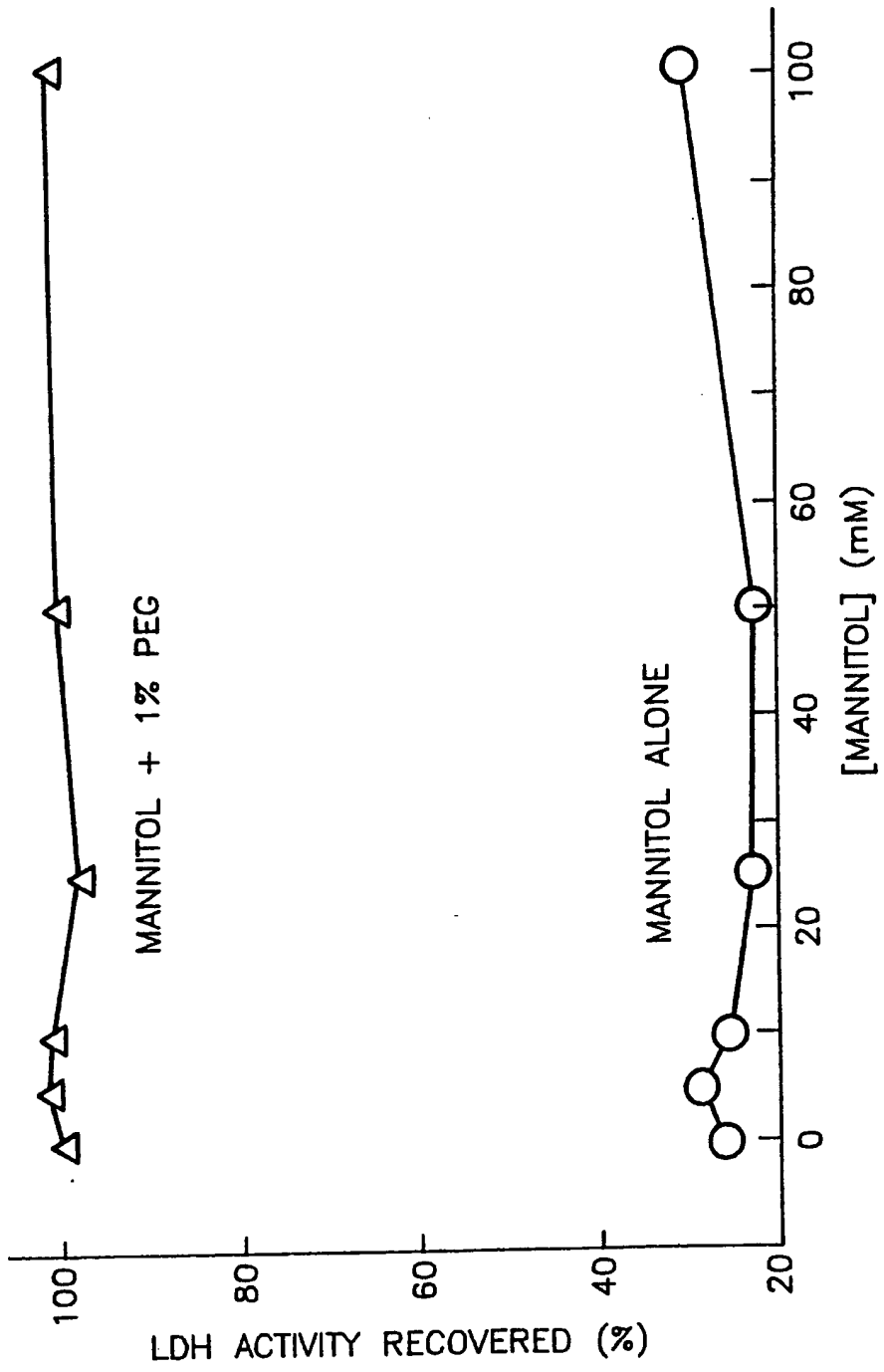
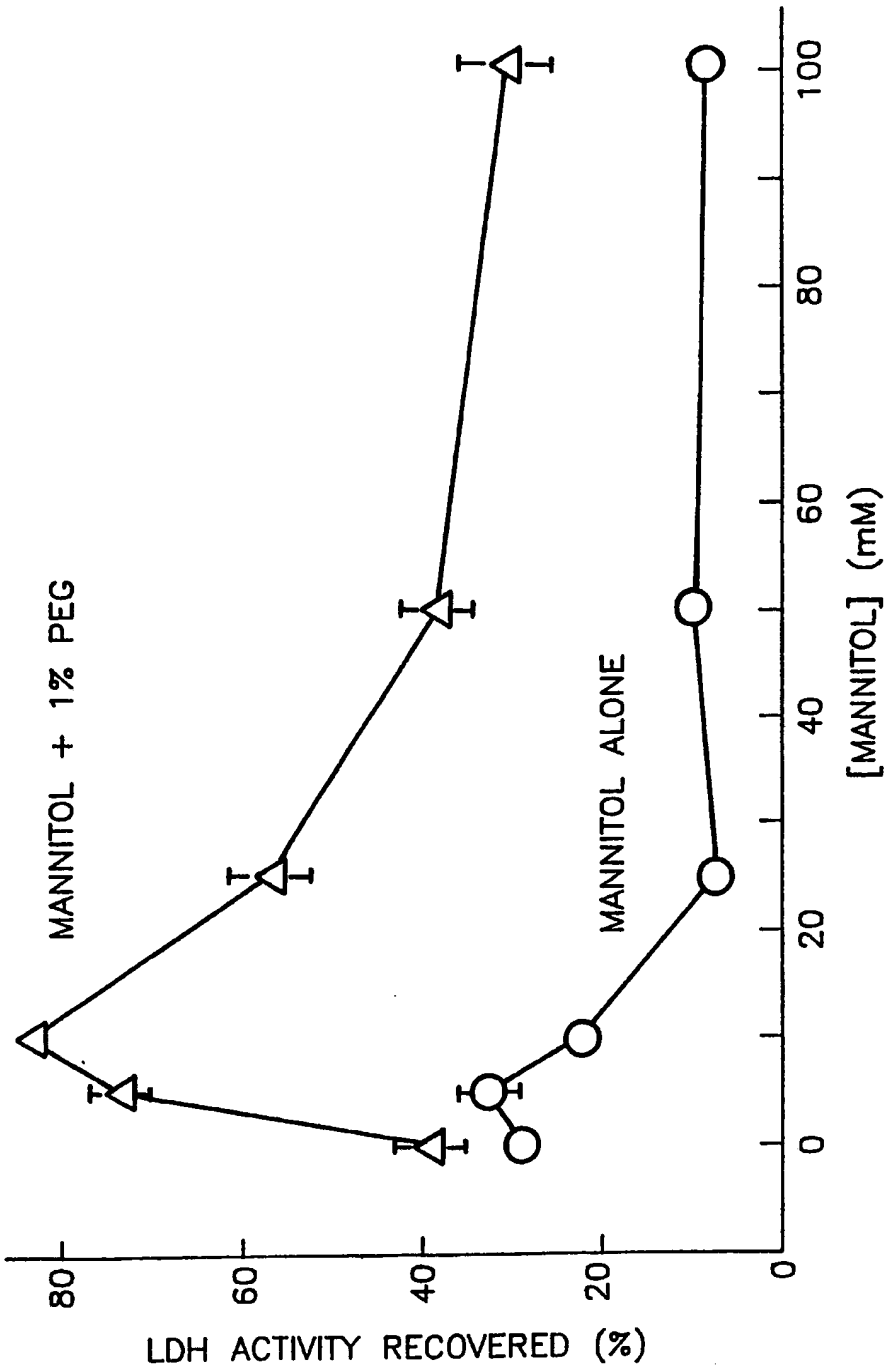

MANNITOL + 1% PEG
MANNITOL ALONE
LDH ACTIVITY RECOVERED (%)
100
80
60
40
20
0
20
40
60
80
100
[MANNITOL] (mM)

FIG. 5B


MANNITOL + 1% PEG
MANNITOL ALONE
LDH ACTIVITY RECOVERED (%)
[MANNITOL] (mM)
0
20
40
60
80
100

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05643

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :435/1,2,188
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/1,2,188

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US, A, 4,874,690 (Goodrich, Jr. et al.) 17 October 1989, see Table IV.	15-17,30-33 1-33
X	Transplantation, volume 49, number 2, issued February 1990, W. Wicomb et al. et al., "Optimal Cardioplegia and 24-Hour Heart Storage with Simplified UW Solution Containing Polyethylene Glycol", pages 261-264, see Table I	17,23,24,26,30-33
X	US, A, 3,177,117 (Saunders) 06 April 1965, see claim 4.	30-33
X Y	PCT, A, WO 89/09610 (Hora et al.) 19 October 1989, see Table I.	30-33 1-33
X Y	US, A, 4,439,421 (Hooper et al.) 27 May 1984, see example I.	17,19,21-27,30,32,33 1-33
X	US, A, 4,574,081 (Shymon) 04 May 1986, see claims 1-5.	17,22-24,2628-30,32,33
Y	US, A, 4,931,385 (Block et al.) 05 June 1990	1-33

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 July 1992	Date of mailing of the international search report 02 NOV 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer SANDRA SAUCIER Telephone No. (703) 308-1084

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05643

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal Diary Science, volume 73, Number 12, 1-33 issued 1990, Carpenter et al., "Comparison of Solute-Induced Protein Stabilization in Aqueous Solution and in the Frozen and Dried States", pages 3627-36, see Tables 3 and 4.	1-33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05643

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A01N 1/02
C12N 9/96

93-029769/04 B07 GREC 91.05.31
GREEN CROSS CORP *JP 04354954-A
91.05.31 91JP-157682 (92.12.09) A61M 5/24, A61K 9/127

Injection syringe packed with medicinal liposome - in which freeze dried agent of liposome is packed in discharge side chamber

C93-013293

B(11-C2, 12-M11F)

In an injection syringe, a freeze dried agent composed of liposome of medicine is packed in the discharge side chamber of liq. medicine and chambers packed with a solvent to dissolve a medicine are provided for the opposite position of the discharge port side of liq. medicine of the chamber.

USE/ADVANTAGE - The injection syringe packed with the liposome of medicine such as freeze-dried medicines can be simply operated in use without danger of inter-mixt. of miscellaneous bacteria during dissolving and also can lessen losses of small amts. of medicine when diluted with water. The use of the syringe enables one to quickly administrate freeze dried medicine composed of liposome of medicine. (7pp Dwg.No.0/0)

92-330092/40 B07 STEF/ 89.11.15
STEFANOV A V *SU 1699343-A3
89.11.15 895U-4785619 *(91.12.15)* A61K 9/127

Synthesis of anti-hypoxic prepn. in liposome form - by drying phosphatidyl choline, emulsifying in water, exposing to ultrasound, filtering and lyophilising

C92-146764

Addnl. Data: STEFANOV A V, BRYGINSKII S A, LISHKO V K

B(5-B1P, 12-K6, 12-M11F)

An alcoholic soln. of natural or synthetic phosphatidyl choline is dried in vacuum of an inert N2 atmos. After emulsifying the phospholipid film in an aq. medium with a lipid:water ratio of 1:20-40, the emulsion is exposed to ultrasound for 40 min. at frequency of 44 kHz. The resultant suspension then undergoes sterilisation filtration and lyophilisation.

After two doses (2mg/100g) of the agent administered by inhalation to test animals with experimentally-induced pneumonia, O2 pressure in arterial blood had normalised. Lactic acid content also declined, as did the malonic dialdehyde level of the blood. Unlike the known agent, which cannot be kept for more than 2 hr., the liposome prepn. is stable.

USE/ADVANTAGE - A more stable antihypoxic prepn. in liposome form. Bul.46/15.12.91 (3pp Dwg.No.0/0)